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Free fatty acid levels in fluid of dominant follicles at the preferred insemination time in dairy cows are not affected by early postpartum fatty acid stress

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ABSTRACT

The fertility of high-yielding dairy cows has declined during the last 3 decades, in association with a more profound negative energy balance (NEB) during the early weeks postpartum. One feature of this NEB is a marked elevation in circulating free fatty acid (FFA) concentrations. During the early postpartum period (≤d 42), circulatory FFA levels were measured weekly, and progesterone concentrations and the diameter of the dominant follicles were determined thrice weekly. Retrospectively, cows that ovulated within 35 d postpartum were grouped as "normal ovulating" cows (n = 5), and the others were grouped as "delayed ovulating" cows (n = 5). In both groups, high total FFA levels (>500 μ*M*) were evident immediately postpartum. Interestingly, cows with delayed ovulation had higher plasma FFA concentrations in the first weeks postpartum compared with normal ovulating cows. In both cow groups, FFA decreased to control levels of non-NEB cows within 3 wk postpartum. The FFA compositions and concentrations in fluids from the dominant follicles of postpartum cows were not different between the normal and delayed ovulating cows when measured at potential insemination points: d 55, 80, and 105 postpartum. Interestingly, the concentration of monounsaturated oleic acid was higher and that of saturated stearic acid lower in follicular fluids of both groups compared with that in blood. The level of FFA in follicular fluid was correlated with the ratio of 17β-estradiol (E_2) to progesterone (P_4) in follicular fluid, with a relatively high level of unsaturated FFA in follicles with a low E_2:P_4 ratio. Taken together, these results indicate that a more severe NEB early postpartum is related to a delay in the first postpartum ovulation and does not affect FFA composition in follicular fluid at the preferred insemination time. The high FFA level in dominant follicles with a low E_2:P_4 ratio may be due to a different FFA metabolism in these follicles. The diagnostic value of this observation for selective screening of dominant follicles needs further investigation.

Key words: free fatty acid, dairy cow, follicular fluid, postpartum

INTRODUCTION

The reproductive performance of high-yielding dairy cows has declined markedly during the last 4 decades, from a calving rate of around 55% per insemination in the 1980s to 40% today (Britt, 1992; Sartori et al., 2002; Walters et al., 2002; Butler, 2003; van Knegsel et al., 2005; Diskin et al., 2006). In contrast, the fertility of nonlactating heifers has remained undiminished throughout this period (Sartori et al., 2002). During this period of declining fertility, the milk production of cows has increased appreciably, with the consequence that cows experience a more profound period of negative energy balance (**NEB**) in the early postpartum period. This NEB is considered to be, at least in part, responsible for the decrease in fertility (Britt, 1992; Walters et al., 2002; Butler, 2003; van Knegsel et al., 2005; Kawashima et al., 2012). Cows with a more severe NEB, and consequent more dramatic loss of body condition during the peripartal period, show compromised reproductive performance in terms of delayed resumption in ovarian activity and reduced ovulation rate from the first follicular wave postpartum, compared with cows with less severe body condition loss (Butler and Smith, 1989; Beam and Butler, 1998). A major metabolic characteristic of NEB is the elevation in circulating FFA concentrations. The FFA are released into the circulation from adipose tissue during NEB and periods of glucose deprivation and are transported into albumin complexes to enable uptake by peripheral tissues, where they can be used as an alternative energy source for aerobic functional cells

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(Contreras and Sordillo, 2011). Beyond their role as an alternative energy source, however, elevated levels of FFA (especially saturated FFA) may induce lipotoxic stress in several somatic cell types (Cnop et al., 2001; Listenberger et al., 2001; Maedler et al., 2001; Listenberger et al., 2003; Mishra and Simonson, 2005; Coll et al., 2008; Henique et al., 2010). Granulosa and theca cells may also be adversely affected by elevated FFA levels of, in particular, saturated FFA: in vitro exposure results in reduced cell proliferation and increased apoptosis in both cell types (Mu et al., 2001; Vanholder et al., 2005, 2006). Moreover, exposure of the cumulus–oocyte complex to elevated levels of saturated FFA during final maturation can result in an oocyte with a diminished mitochondrial membrane potential and impaired capacity to develop into a blastocyst (Leroy et al., 2005; Aardema et al., 2011; Wu et al., 2012). These observations indicate that the increase in FFA levels that occurs during NEB may well represent a threat for the maturing follicles and internal structures such as the oocyte.

The main focus of in vivo research has been on the acute effect of elevated FFA levels in blood during NEB on the dominant follicle (Leroy et al., 2005; Aardema et al., 2013a). Information on potential dominant follicles selected around the preferred insemination period is lacking. In general, dairy cows are inseminated from around 60 d postpartum onward to achieve a desired calving interval of 12 to 13 mo (Stevenson, 2007). The sequential growth stages of a presumptive dominant follicle, from the primordial stage until the preovulatory stage, take around 80 d in the cow (Britt, 1992). Initially, during early preantral follicular growth, the follicle contains a single layer of granulosa cells surrounding the oocyte but, during subsequent growth phases, the follicle develops into a structure with several cell layers (Fair et al., 1997). A major function of the follicle is to provide a "blood–follicle barrier" to create a favorable environment for the growing oocyte, which is also demonstrated by the distinct FFA composition between blood and follicular fluid (Leroy et al., 2005; Aardema et al., 2013a). The length of the period of follicular growth and development implies that follicles, which gain dominance at 60 to 80 d postpartum, have been recruited and have started their development in the peripartum period during the NEB. Consequently, these follicles have been fully exposed to the NEB-induced metabolic status and hence elevated FFA levels during the early (preantral) follicular growth stages. It has been hypothesized that exposure of follicles to the unfavorable metabolic conditions of NEB during early follicular growth may have a latent effect on the function of the follicle and hence the quality of its contained oocyte (Spicer and Echternkamp, 1986; Britt, 1992). The NEB condition may consequently contribute to the reduced fertility in high-yielding dairy cows for several weeks to months after the period of the NEB.

In this study, we investigated whether NEB affects FFA composition in follicular fluid of the dominant follicle near the recommended time of insemination. To this end, we determined the concentration and composition of FFA in blood and follicular fluid collected from dominant follicles during the period of postpartum insemination; namely, d 55, 80, and 105 postpartum. Furthermore, the potential effect of elevated FFA levels in the early postpartum period on reproductive activity was investigated by monitoring follicular growth and development and the timing of the first postpartum ovulation. To investigate whether the levels of FFA in follicular fluid were associated with follicular function, we performed a correlation analysis between FFA concentrations and the calculated 17β-estradiol ($\mathbf{E_2}$):progesterone ($\mathbf{P_4}$) ratio from E_2 (parameter for follicular function) and P_4 levels in follicular fluid.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest available purity. Solvents (acetone, acetonitrile, chloroform, methanol, and hexane) were of HPLC grade (Labscan, Dublin, Ireland).

Experimental Procedures on Animals

All animal experiments were approved by Utrecht University's Animal Experimental Procedures Committee. Clinically healthy, pregnant Holstein-Friesian heifers (n = 10) were included in the experiment from the time of calving until d 105 postpartum. The heifers were fed 10 kg of maize, 0.5 kg of soybean hulls, ad libitum grass silage daily, supplemented with 2 kg of concentrate feed (Synchro-optimaal, De Heus Voeders BV, Ede, the Netherlands) on the day of calving, increasing to 8 kg of concentrate on d 14 postcalving until the end of the experiment. The heifers had unlimited access to water and, once a week, their BCS (1–5 scale) was determined. From d 14 postpartum, per rectum ultrasonography was performed 3 times a week using a 240 Parus scanner (Pie Medical, Maastricht, the Netherlands) equipped with a 7.5-MHz linear array transducer, and the diameters of ovarian follicles (>5mm) were recorded. Cows with a first ovulation before d 35 postpartum were grouped as "normally ovulating," and

cows without a first ovulation before d 35 postpartum were grouped as "delayed ovulating." The cut-off point of d 35 was based on previous studies (Gautam et al., 2010; Crowe et al., 2014). The first ovulation was considered to have taken place when 3 successive blood progesterone measurements exceeded 2 ng/mL, combined with ultrasonographic evidence of corpus luteum formation. The follicular fluid was collected individually from follicles ≥14 mm in diameter by transvaginal ultrasound-guided follicle aspiration (Pieterse et al., 1991), on discrete days from d 42 to 105 postpartum. Follicular fluid from individual follicles was centrifuged for 10 min at 3,000 × *g* at 4°C and the supernatant was stored at −80°C. Blood for progesterone analysis was collected 3 times a week from the jugular vein using a Vacutainer system and heparin-coated tubes (Becton Dickinson and Co., Franklin Lakes, NJ). The concentrations of E_2 and P_4 in follicular fluid were determined in aliquots of 1 to 25 μL of fluid, depending on the type of hormone and size of the follicle. Follicular fluid was analyzed using solid-phase [^{125}I] RIA (Coat-A-Count; TKE2 and TKPG respectively; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as described previously (Aardema et al., 2013a). Serum samples (serum tubes; Becton Dickinson and Co.) for total FFA analysis were collected weekly from the day of parturition until d 42 (FA 115 kit; Randox Laboratories Ltd., Crumlin, UK).

Extraction and Isolation of FFA

The serum and follicular fluid samples collected on d 7 (serum only), 55, 80, and 105 postcalving were selected to isolate and analyze the FFA fraction, as described previously (Aardema et al., 2013a). The total lipid fraction from 100 μL of blood or follicular fluid was extracted according to the method of Bligh and Dyer (1959) and evaporated under a constant stream of nitrogen gas at 40°C. Deuterated palmitic acid [7,7,8,8-$^2H_4$] (Cambridge Isotopes Laboratories Inc., Cambridge, MA) was added as an internal standard to the samples (10 nmol/sample) at the beginning of the lipid extraction procedure to allow calculation of recovery efficiency and absolute concentrations. Isolation of the FFA fraction was performed as described by Kates (1986), but without the heating step and maintaining the samples on ice to avoid hydrolysis of the FFA. The protocol was as follows: 1 mL of 0.3 *M* NaOH in methanol:water (9:1, vol/vol) was added to the evaporated lipid mixture, and the polar phase was washed 3 times with 1 mL of hexane to purify the FFA fraction in the resulting aqueous methanol phase. This was subsequently acidified and the FFA was extracted using 3 portions of 1 mL of hexane, evaporated under a constant stream of nitrogen gas at 40°C, and stored in an atmosphere of 100% nitrogen at −20°C until analysis.

Analysis of FFA by HPLC Mass Spectrometry

Free fatty acids were dissolved in 100 μL of methanol:acetonitrile:chloroform:water (46:20:17:17, vol/vol/vol/vol) and injected onto a Halo C18 (150 × 3.0 mm; particle size of 2.7 μm) HPLC column (Advanced Material Technology Inc., Wilmington, DE). The injection volume was 40 μL and the temperature of the column was maintained at 40°C. Lipids were eluted using a linear gradient, from acetonitrile:methanol:water (6:9:5, vol/vol/vol) 2.5 m*M* ammonium acetate to acetone:methanol (4:6, vol/vol) 2.5 m*M* ammonium acetate for 15 min, followed by isocratic elution with the latter solvent for 10 min and regeneration of the column for 5 min, all at a flow rate of 0.6 mL/min. Mass spectrometry of FFA was performed using electrospray ionization (ESI) on a 2000 QTRAP system (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Source temperature was set to 450°C and nitrogen was used as curtain gas. The declustering potential was set to −40 V. Full scans were performed in negative mode in the mass:charge (m/z) range from 225 to 400 amu. Peaks were identified by comparison of retention time and mass spectra with authentic standards, and calibration curves were generated to correct for differences in response factors.

Data Processing

Lipid data were recorded using Analyst software (version 1.4.2; MDS Sciex, Concord, ON, Canada) and exported in mzXML format. Peak detection, integration, and alignment were performed using the open-source software package XCMS, running under R statistical software (Smith et al., 2006). A correlation matrix of the combined data sets was calculated in R. Principal component discriminant analysis was also performed with R using Pareto scaling, in which the value for each peak is transformed by subtraction of the mean for all samples and division by the square root of the standard deviation. In this way, data maintain a dimension (units), and peaks with a good signal-to-noise ratio will gain importance without allowing intense peaks to dominate the analysis.

Statistical Analysis

The statistical analysis was performed using R statistical software (version 3.0.2; R Development Core Team, 2013). Longitudinal comparison of the total

FFA concentrations and comparison of the individual FFA concentrations in blood and follicular fluid across the groups were performed using a linear mixed model (Pinheiro et al., 2011; R package version 3.1–101 'nlme') with days postpartum and group as fixed effects and cow as a random effect. For the longitudinal comparison of blood and follicular fluid FFA concentrations, a linear mixed model (Pinheiro et al., 2011; R package version 3.1–101 'nlme') was applied with days postpartum and blood and follicular fluid as full factorial fixed effects and animal as a random effect, so that the correlation between observations was accounted for. Statistical analysis of the E_2 and P_4 concentrations, E_2:P_4 ratio, and FFA level in follicular fluid was performed by nonparametric correlation (Spearman r) using GraphPad Prism for Windows (version 5.02; GraphPad Software, San Diego, CA; www.graphpad.com). Unless stated otherwise, all measures are reported as mean ± standard deviations; $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Cows with Delayed Ovulation Have Higher Plasma Total FFA Concentrations

The total FFA concentrations in the blood plasma of 10 cows were followed weekly during the first 6 wk postpartum. In addition, time of ovulation was monitored 3 times a week by rectal ultrasound detection of follicle size and collapse, and by detection of elevated progesterone concentrations in the blood. Half of the cows had a first postpartum ovulation within 35 d of calving (Figure 1A–E) and were grouped as normally ovulating cows, whereas the other 5 cows showed a first ovulation after 35 d postpartum (Figure 1F–I) and were assigned to the delayed ovulation group. Both groups showed follicular growth from as early as 14 d postpartum, the time of the first postpartum ultrasound examination, and developed follicles to a size compatible with ovulation (Figure 1). However, most of the dominant follicles in the delayed ovulation group became atretic (Figure 1F–I). Interestingly, 2 out of 5 cows in the group with delayed ovulation developed follicles with a larger diameter than the follicles ovulated before d 35 in the normal group (Figure 1). One cow developed a luteal cyst (Figure 1F). Cows with a delayed first postpartum ovulation had overall significantly higher total FFA concentrations in blood during the early postpartum period than the cows with a first ovulation before d 35 ($P < 0.01$; Figure 2A). The significant time by group effect, is indicated by the light gray (yellow) and dark gray (purple) lines in Figure 2A. The rate of the concentration decrease (K; for the formula of the lines, see Figure 2 caption) was comparable among the groups, and the overall higher concentration of FFA in the group with a delayed ovulation was due to higher starting values of FFA (Figure 2A). We detected no significant differences in FFA levels between groups at individual time points. The main differences in the FFA concentrations among the groups were at d 0, 7, and 14; for delayed ovulating cows, the mean (±SD) total FFA concentration was 988 ± 297, 600 ± 307, and 500 ± 121 μM compared with 732 ± 207, 424 ± 167, and 280 ± 74 μM for cows that ovulated before d 35 (Figure 2A). The composition of FFA molecular species in the blood at d 7 postpartum was comparable in both groups (Figure 2B), indicating that the increase in total FFA in the delayed ovulating group was primarily a factor of accelerated mobilization of body fat, rather than mobilization of specific FA-containing lipid pools (Figure 2B). From 21 d postpartum, the FFA concentrations in both groups returned to baseline levels (around 250 μM, Figure 2A).

Normal and Delayed Ovulating Cows Have Similar FFA Compositions in Blood and Follicular Fluid

In most commercial breeding programs, dairy cows are not inseminated before 55 d postpartum. For this reason, we monitored blood FFA levels and composition in cows with a normal first ovulation and those with a delayed first ovulation at 55, 80, and 105 d postpartum. The average amount of FFA was comparable at the different time points (Supplementary Figure S1; http://dx.doi.org/10.3168/jds.2014-7970); therefore, the average amount of FFA during time is grouped in one graph (Figure 3A). The groups did not differ in serum FFA concentrations or molecular species composition at d 55, 80, and 105 postpartum ($P = 0.62$, $P = 0.54$, and $P = 0.79$). Likewise, the fluid recovered from dominant follicles on d 55, 80, and 105 postpartum was comparable at the different time points and showed no differences in FFA concentrations or molecular species composition between normal and delayed first postpartum ovulation cow groups ($P = 0.36$; $P = 0.83$, and $P = 0.21$ for, respectively, d 55, 80, and 105 postpartum; Figure 3B and Supplementary Figure S2; http://dx.doi.org/10.3168/jds.2014-7970).

FFA Levels and Molecular Species Composition of Blood Differ from that in Follicular Fluid

Interestingly, in both normal and delayed ovulation cows, we noted a marked difference in the relative concentrations and most prominent FFA molecular species (≥5% of total FFA) between blood and follicular fluid of FA with a chain length of 18 carbon atoms (Fig-

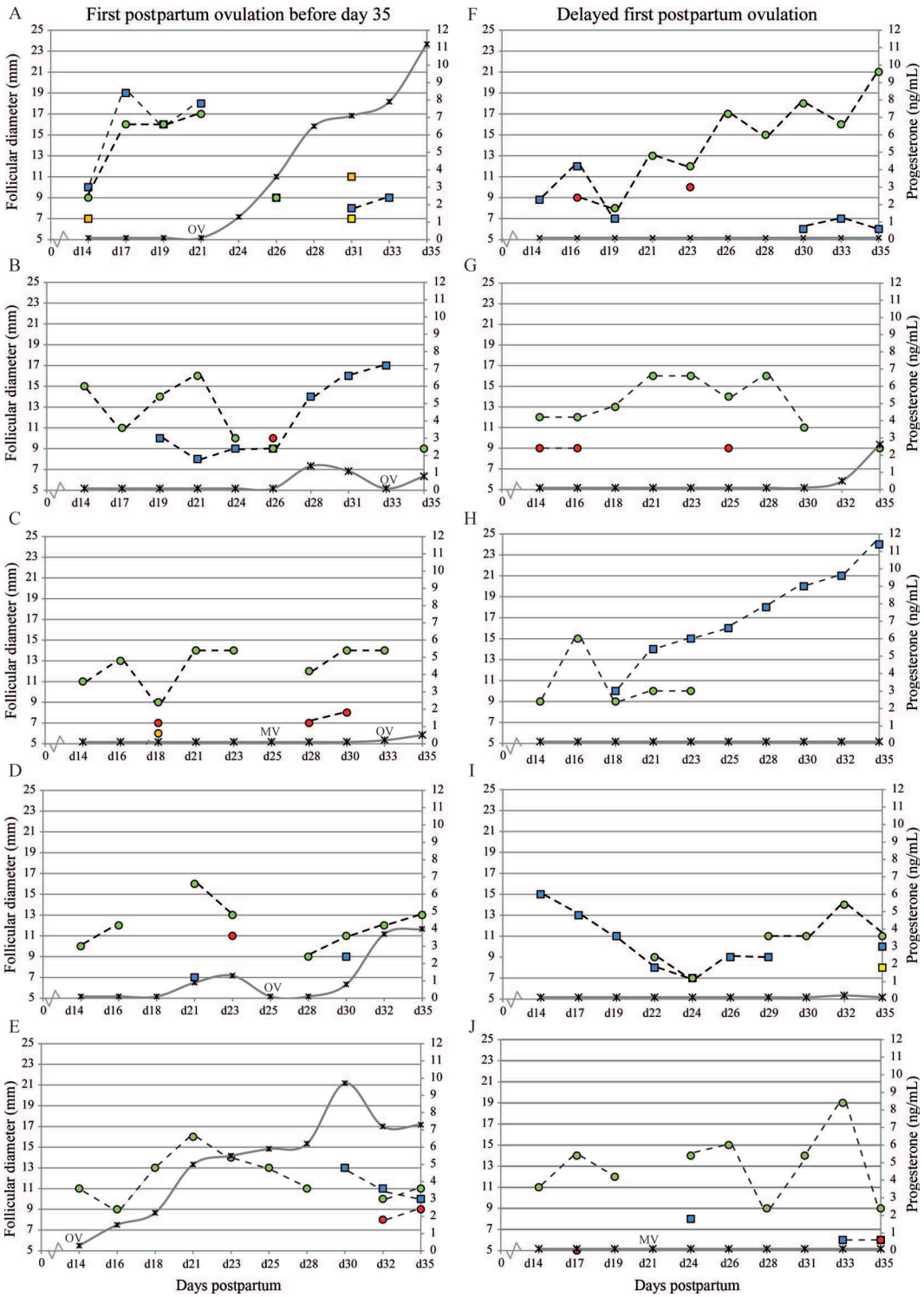

Figure 1. Ovarian follicular growth patterns during the early postpartum period (d 14–35): diameter of follicles present on the left (squares) and right (circles) ovaries in cows that had their first postpartum ovulation before (A–E) or after d 35 postpartum (F–J). The slight progesterone increase (gray line) in panels B and C was followed by 3 subsequent measurements with a progesterone value of ≥2 ng/mL and resulted in an estimated time of ovulation before d 35; the cow in panel G had a low value of progesterone at the subsequent measurement after d 35 and was defined as delayed ovulating cow. The moment of ovulation was retrospectively defined and based on at least 3 subsequent measurements with a progesterone value of ≥2 ng/mL. Day of parturition is $t = 0$. Per rectum ultrasound was performed thrice weekly on Monday, Wednesday, and Friday. Note that the y-axis starts from a diameter of 5 mm. OV = estimated day of ovulation; MV = missing value. Color version available online.

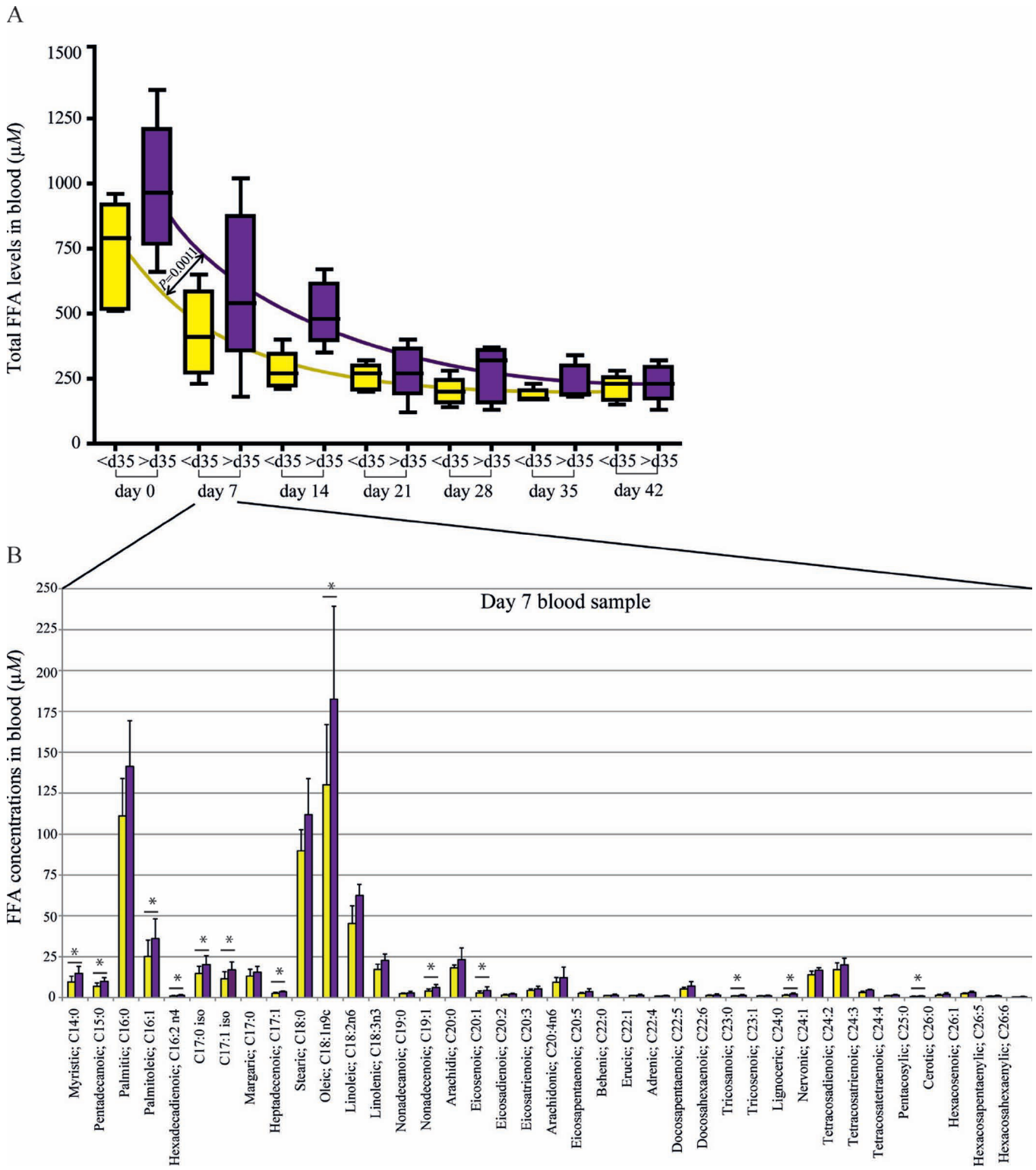

Figure 2. Total FFA concentration in blood and follicular fluid of postpartum cows. The total FFA concentrations in blood from d 0, the day of parturition, until d 42 (A) and FFA profile at d 7 postpartum (B) are presented for cows with a first ovulation before d 35 postpartum [light gray (yellow)] and with a delayed first ovulation postpartum [dark gray (purple)]. The boxes in panel A represent the median (horizontal line in the middle of the boxes) and interquartile ranges, the bottom and top of the box indicate the lower and upper quartiles. The whiskers include all cases. The data were fitted to a line with the formula [FFA] = base level + [($FFA_{t=0}$) – base level] × exp[K×(t_{day})] as shown by the light gray (yellow) and dark gray (purple) lines in panel A. In panel B, the values are means ± SD. Asterisks indicate a significant difference ($P < 0.05$). Color version available online.

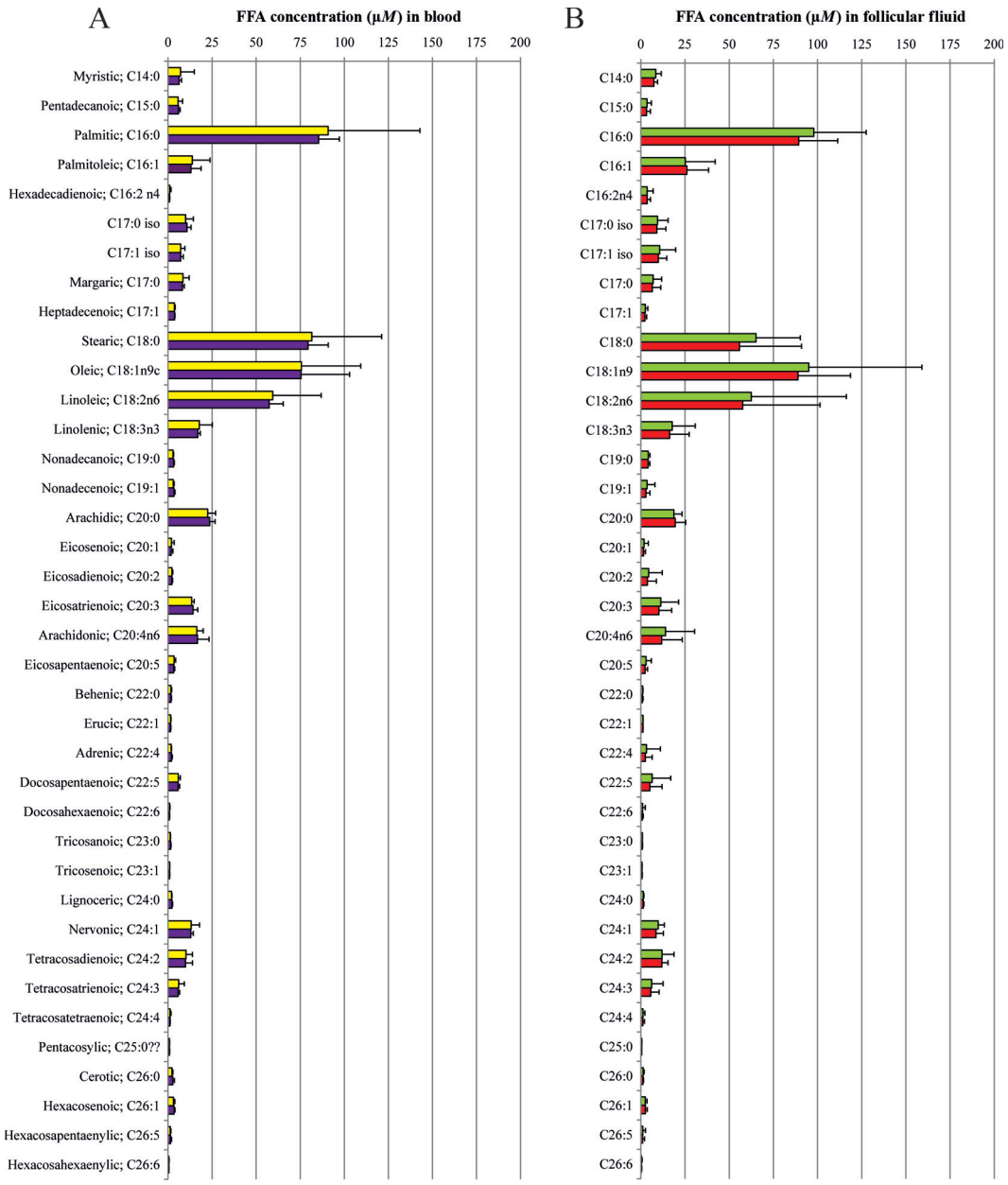

Figure 3. The FFA composition in blood and follicular fluid of postpartum dairy cows. The average of the absolute concentrations of individual FFA at d 55, 80, and 105 is shown for cows with a first ovulation before d 35 [light gray (yellow and green)], and cows with a delayed first ovulation postpartum [dark gray (purple and red)] in blood (A) and follicular fluid (B). Values are means ± SD. Color version available online.

ure 4) and 16 carbon atoms (Figure 5). Stearic acid (C18:0) concentrations in follicular fluid were lower than in blood in both normal ($P < 0.05$; Figure 4A) and delayed ovulating cows ($P < 0.001$; Figure 4B). In contrast, significantly higher concentrations of oleic acid (C18:1) and palmitoleic acid (C16:1) were detected in follicular fluid compared with blood in both normal ($P < 0.05$; Figure 4C and $P < 0.001$; Figure 5C) and delayed ovulating cows ($P < 0.05$; Figure 4D and $P < 0.001$; Figure 5D). We detected a concomitant and significant increase in desaturation index for C18 (Figure 4G,H) and C16 FFA molecular species (Figure 5E,F) in follicular fluid compared with the corresponding blood samples.

Principal Component Analysis Reveals Distinct FFA Composition of Follicular Fluid and Blood

A principal component analysis (**PCA**) was performed on the FFA composition in corresponding blood

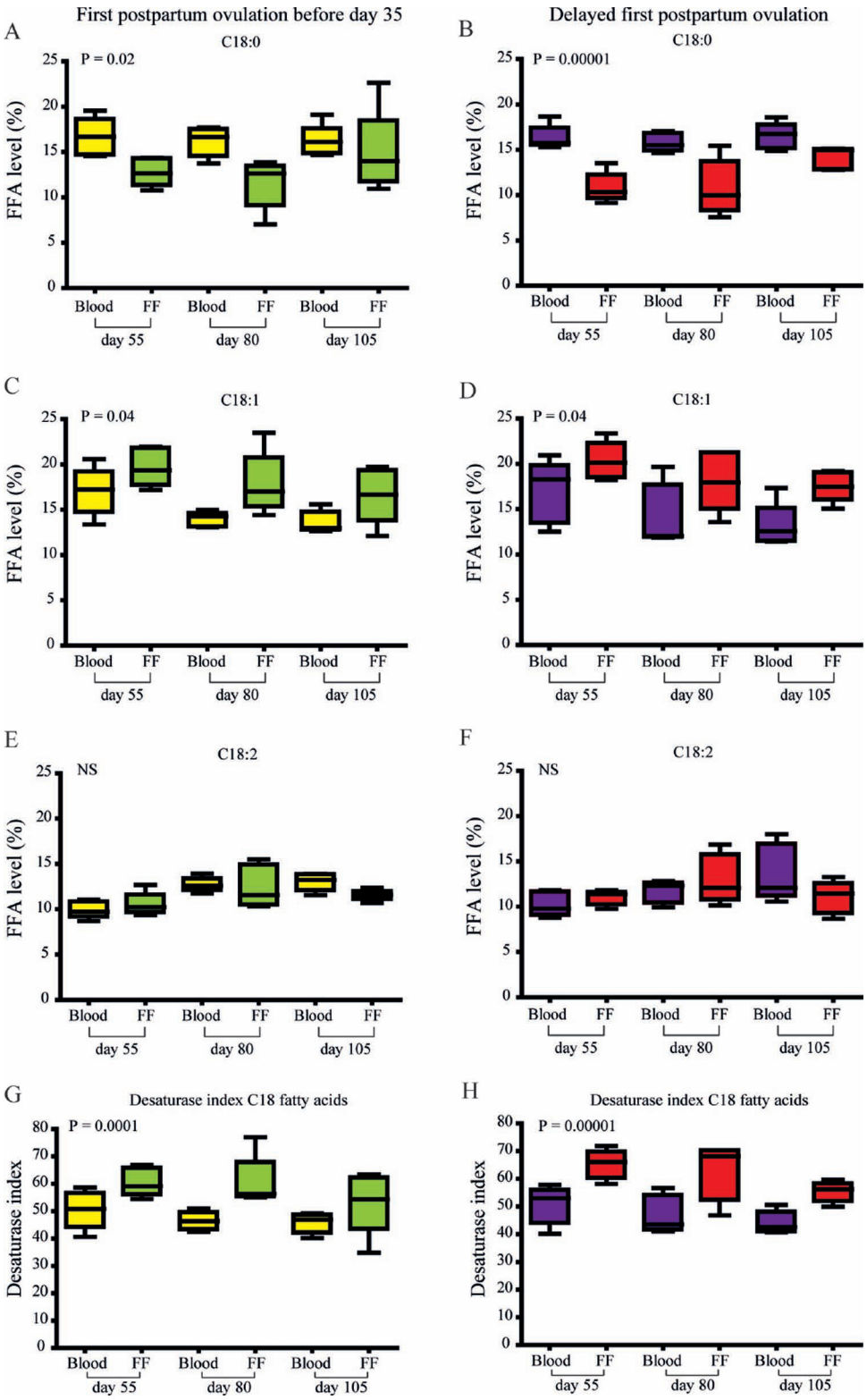

Figure 4. Box and whisker plots for the relative amount of C18 FFA in blood and follicular fluid. The relative values of FFA in blood [light gray (yellow)] and follicular fluid [gray (green)] of normally ovulating cows (panels A, C, and E) compared with blood [dark gray (purple)] and follicular fluid [gray (red)] from cows with a delayed first postpartum ovulation (panels B, D, and F); saturated C18:0 (panels A, B), monounsaturated C18:1 (panels C, D), and polyunsaturated linoleic acid (panels E, F). The desaturation index of C18 FFA (C18:1/C18:1 + C18:0 × 100) in blood and follicular fluid is shown for early (G) and delayed ovulators (H). The *P*-values represent an overall significant difference between blood and follicular fluid. Color version available online.

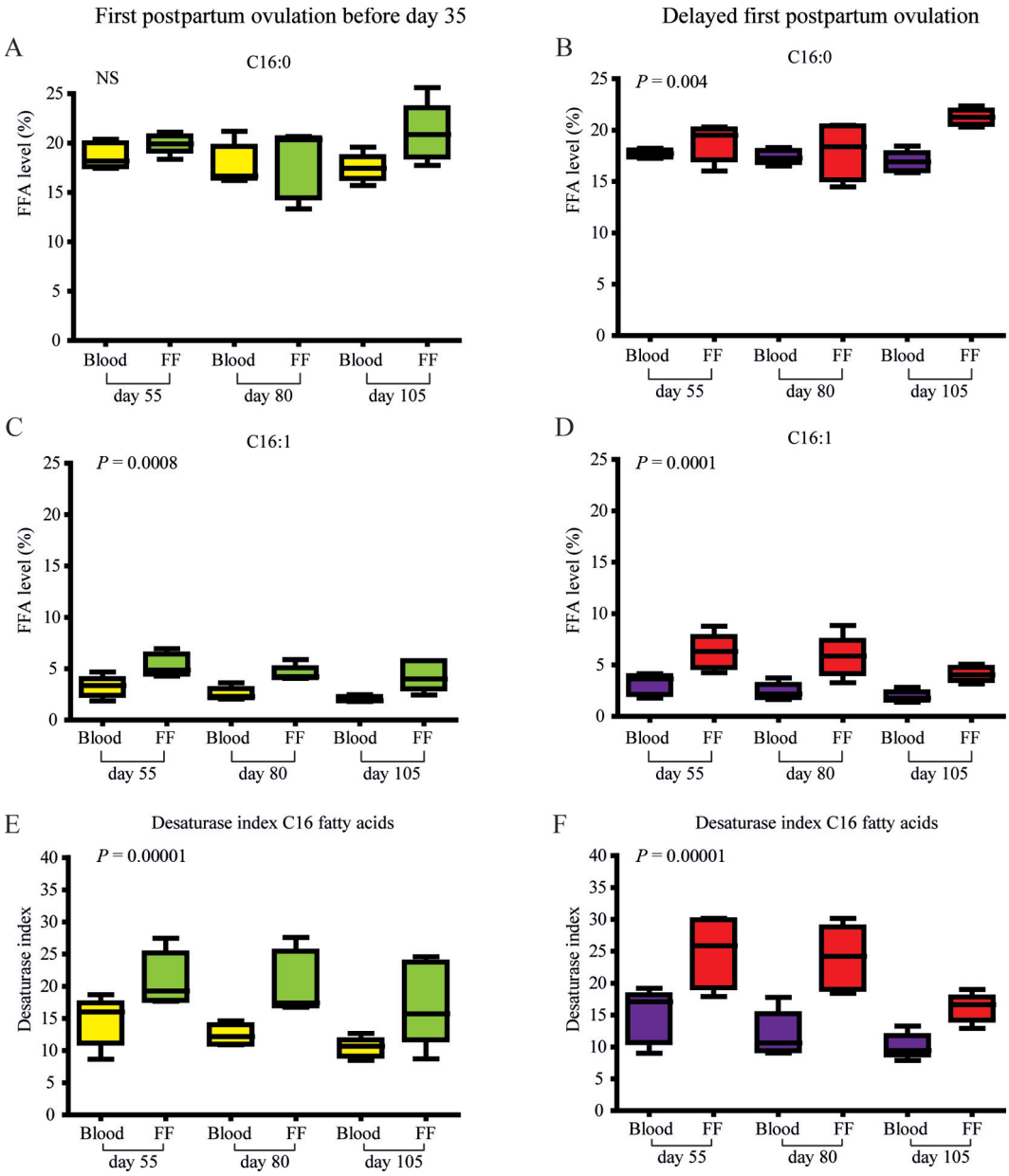

Figure 5. Box and whisker plots for the relative amount of C16 FFA in blood and follicular fluid. The relative values of FFA in blood [light gray (yellow)] and follicular fluid [gray (green)] of normally ovulating cows (panels A and C) compared with blood [dark gray (purple)] and follicular fluid [gray (red)] of cows with a delayed first postpartum ovulation (panels B, D); saturated palmitic acid (C16:0, A, B) and mono-unsaturated C16:1 (panels C, D). The desaturation index of C16 FFA (C16:1/C16:1 + C16:0 × 100) in blood and follicular fluid is shown for normal (E) and delayed ovulators (F). The *P*-values represent an overall significant difference between blood and follicular fluid. Color version available online.

and follicular fluid samples. The PCA revealed distinct clustering of blood and follicular fluid samples, which further indicated the differences in composition between blood and follicular fluid (Figure 6). Interestingly, together with the previously noted differences in C18 FFA between follicular fluid and blood, less-abundant FFA molecular species such as polyunsaturated C20 and C24 FFA as well as odd-chain-length (C17) FFA were shown to be valuable trace species for predicting differences between follicular fluid and blood. Of special interest was the fact that the difference between follicular fluid and corresponding blood samples ($P = 0.0014$) was time independent and remained unchanged over the 55, 80 and 105 d postpartum sampling points

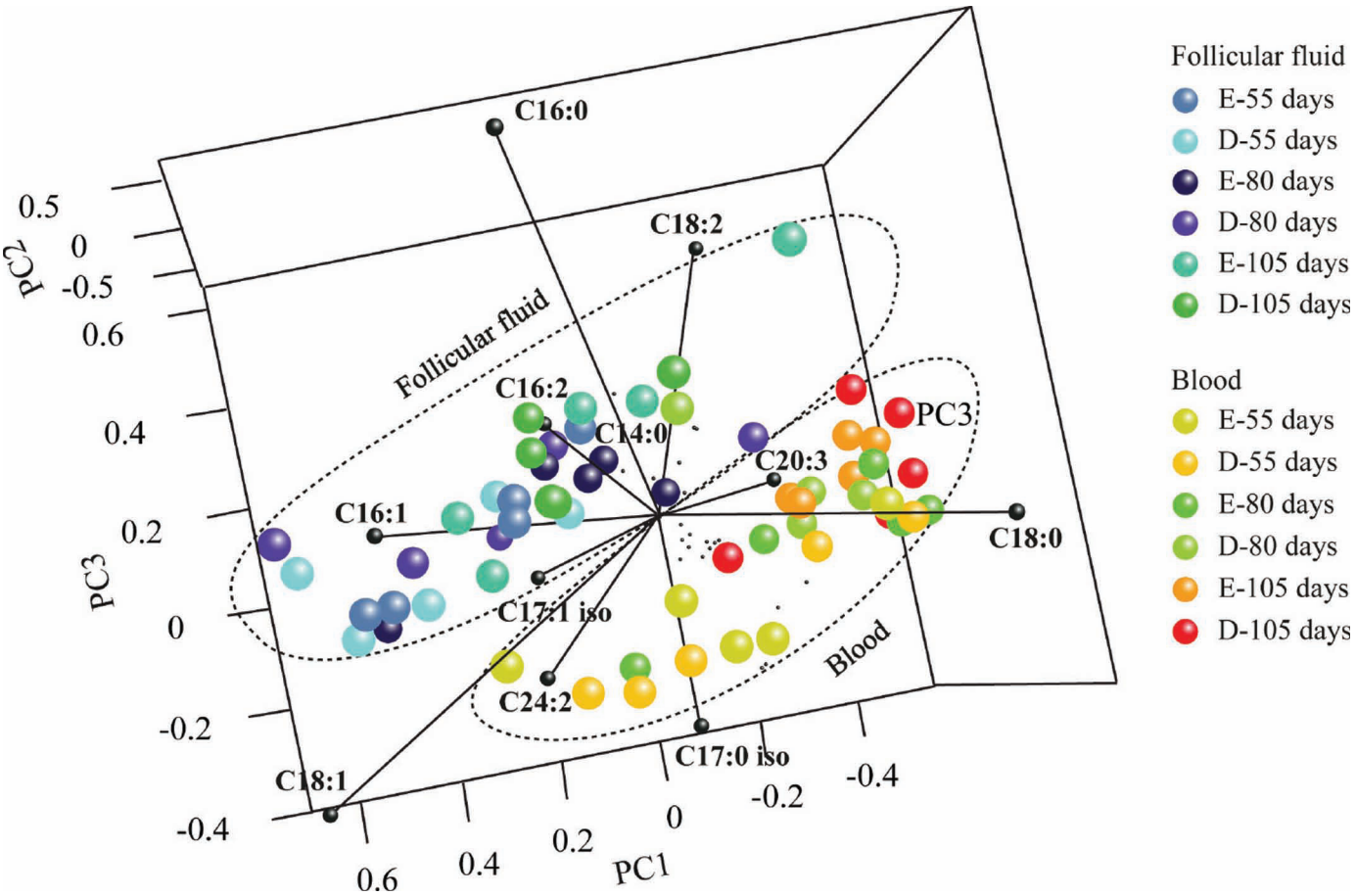

Figure 6. Principal component analysis (PCA) of FFA composition in blood and follicular fluid. The PCA revealed consistently distinct FFA compositions in blood and follicular fluid, as shown by the different clustering in the score plot of blood and follicular fluid samples from cows with an early (E) and delayed (D) first postpartum ovulation. The major contributors to the distinct FFA compositions were saturated C18:0 and polyunsaturated linoleic acid (C18:2), as they had coordinates with the highest values in the loading plot. Fatty acids with PCA loading values outside the 75% quartile range were plotted as black spheres; FA within this range were plotted as small gray spheres.

($P < 0.00001$, $P = 0.027$, and $P = 0.0029$, respectively). Again, no differences were found between cows with a normal versus a delayed first postpartum ovulation ($P = 0.44$).

A Low E_2:P_4 Ratio Is Related to FFA Changes in the Follicle

The concentrations of E_2 and P_4 in follicular fluid of cows from both groups and among the different time points were not different. The concentrations of E_2 and P_4 were (mean ± SD) 2.5 ± 2.2 and 0.86 ± 2.3 μM, respectively, for cows with an early ovulation and 1.4 ± 1.2 and 0.4 ± 0.5 μM, respectively, for cows with a delayed first postpartum ovulation. The concentration of E_2 was correlated with the level of FFA in follicular fluid (r = −0.67; Figure 7A), whereas no correlation was found for P4 concentration and FFA level (r = −0.099; Figure 7B). Interestingly, a low E_2:P_4 ratio in follicular fluid of dominant follicles was correlated with increased total FFA levels in follicular fluid (Figure 7C). The E_2:P_4 ratio correlated with the total FFA levels in follicular fluid (r = −0.64, $P < 0.001$; Figure 7C) and was, respectively, r = −0.55 for saturated FFA ($P < 0.05$; Figure 7D), r = −0.63 for monounsaturated FFA ($P < 0.001$; Figure 7E), and r = −0.61 for polyunsaturated FFA ($P < 0.001$; Figure 7F). The higher level of FFA in follicles with a low E_2:P_4 ratio was in particular due to substantially higher levels of monounsaturated FFA and polyunsaturated FFA (Figure 7E and F). Individual molecular FFA species of the saturated, monounsaturated, and polyunsaturated subgroups all gave comparable correlations of FFA levels versus E_2:P_4 ratio (data not shown). The increased FFA levels in follicular fluid were not observed in their corresponding blood samples (Figure 7).

DISCUSSION

This study demonstrates that a significantly higher level of FFA in the circulation during the early postpartum period, and thus a more severe NEB of the cow, was related to a delayed first postpartum ovulation (>35 d postpartum).

Early resumption of ovarian activity and an early first postpartum ovulation have generally been considered useful indicators to predict fertility of the cow in the subsequent period, with ovulation after d 35 being associated with reduced fertility in the postpartum period (Staples et al., 1990; Gautam et al., 2010; Galvão et al., 2010; Crowe et al., 2014). Cows with delayed ovulation are less likely to conceive on first AI and before 100 d postpartum (Gautam et al., 2010). Cows with nonovulatory first-wave follicles experience a longer interval to the day of the energy balance nadir, which was calculated from daily net energy intake and loss (Butler and Smith, 1989; Beam and Butler, 1998). The 3 potential fates of a presumptive dominant follicle are ovulation, atresia (regression), or cyst formation (Peter et al., 2009). In the delayed ovulation group, most of the dominant follicles became atretic in the early postpartum period and a luteal cyst was formed in one of the cows. Interestingly, despite time of ovulation, cows in both the normal and delayed ovulation groups developed dominant follicles to a preovulatory size before d 35 postpartum.

At 2 wk postpartum, cows with high blood levels of FFA (>500 μM) showed delayed ovulation, whereas cows in which the levels decreased within the first week postpartum to <500 μM had a first postpartum ovulation before d 35. The levels of FFA in blood reported here are in line with earlier studies of postpartum dairy cows (Rukkwamsuk et al., 1998; Leroy et al., 2005). Previous in vitro data reported that levels of saturated FFA, comparable to the levels in d-7 blood samples in this study, induced apoptosis in theca and granulosa cells and resulted in reduced cell numbers (Mu et al., 2001; Vanholder et al., 2005, 2006). Elevated FFA levels during NEB in the early postpartum period may thus hamper granulosa and theca cells of early-stage follicles and result in subsequent malfunctioning of the follicle and potentially the blood–follicle barrier and unfavorable conditions for the oocyte.

The preferred time of insemination in dairy cows is optimally around 55 d postpartum to ensure the desired intercalving interval of 12 to 13 mo (Stevenson, 2007). At this longer time interval postpartum (from d 35 onward), the blood FFA levels in both groups of cows normalized to 250 μM. However, follicles that gain dominance around the preferred insemination time will have experienced NEB during the early stages of follicular development, as the process of follicular growth is estimated to take around 60 to 80 d in cows (Spicer and Echternkamp, 1986; Britt, 1992). In relation to this, it has been hypothesized (Spicer and Echternkamp, 1986; Britt, 1992) that oocytes from dominant follicles exposed to NEB during early development suffer from impaired developmental competence, a phenomenon that could explain the relation between more severe NEB and reduced fertility. In this study, we tested whether differences in FFA stress imposed in the first weeks postpartum had effects on FFA composition and abundance in the dominant follicle fluids of the 2 cow groups with normal and delayed ovulation. An effect of NEB on the FFA composition in follicular fluid near the time of insemination could be expected to have broad consequences for the quality of the oocyte. Indeed, previous in vitro experiments demonstrated a detrimental effect of elevated levels of, in particular, saturated FFA on the developmental competence of the oocyte (Leroy et al., 2005; Aardema et al., 2011; Wu et al., 2012). However, FFA levels found in follicular fluid of both cow groups were recovered and did not exceed post-NEB blood FFA levels at the preferred insemination time.

Marked differences in FFA molecular species compositions between blood and corresponding follicular fluid samples were evident in both groups of cows, independent of sampling time postpartum. The distinct FFA molecular species composition of blood and follicular fluid indicates that the blood–follicle barrier was functional for all dominant follicles observed. Thus, NEB appears to have no effect on FFA levels in follicular fluid of dominant follicles at a subsequent time during the preferred insemination period. We note here that this study did not investigate the effect of elevated FFA on the oocytes in early-stage follicles that are exposed to NEB and assume dominance in the preferred time-window for insemination. Future research should investigate the effect of the early postpartum elevation in FFA concentrations on the oocytes present in early-stage preantral follicles.

The different FFA composition of blood and follicular fluid may have derived from selective transport of FFA through the blood–follicle barrier, subsequent intrafollicular metabolism, or storage in the cells that line the follicle. For instance, theca and granulosa cells may, by means of the enzyme stearoyl-CoA-desaturase, convert saturated FFA into monounsaturated FFA by dehydrogenation at the Δ^9 carbon chain position of C18 and C16 fatty acids. Indeed, stearoyl-CoA-desaturase has been identified in granulosa and cumulus cells of the rat (Moreau et al., 2006) and may have contributed to the distinct composition of FFA in follicular fluid compared

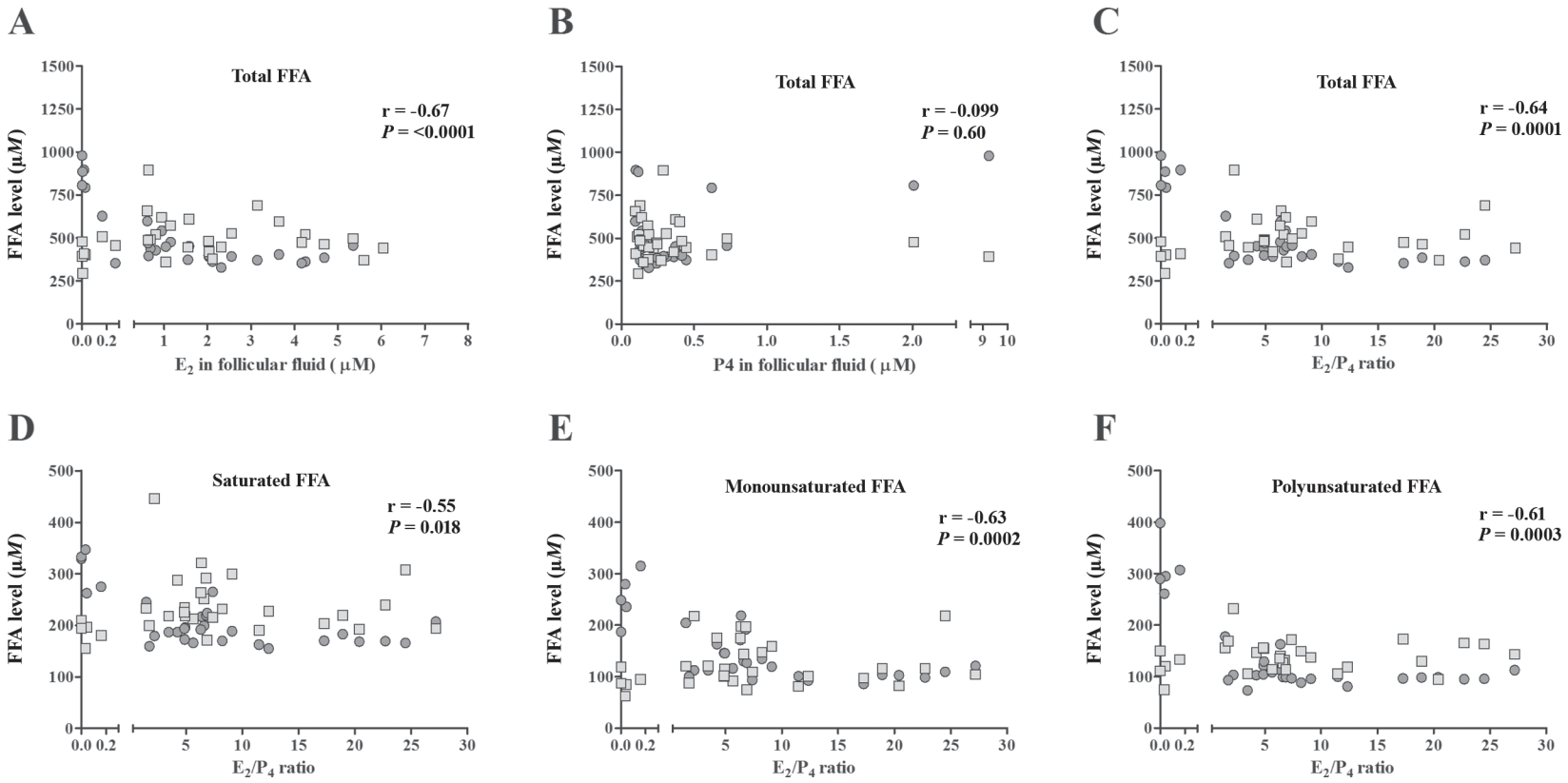

Figure 7. Concentrations of estradiol (E_2) and progesterone (P_4), E_2:P_4 ratio, and FFA level in fluid of dominant follicles. The E_2 and P_4 concentrations and their correlation with total FFA levels in follicular fluid (circles) are presented in panels A and B, respectively. The correlations of E_2:P_4 ratio with total, saturated, monounsaturated, and polyunsaturated FFA levels in fluid of dominant follicles is presented in panels C, D, E, and F, respectively. To compare FFA levels in follicular fluid with levels in blood, corresponding blood samples are represented by squares. The r-values and *P*-values refer to the correlation between the E_2:P_4 ratio and FFA levels in follicular fluid. Note the different x-axis scale in panels A to C. The concentrations of E_2 and P_4 and the ratio of E_2:P_4 were low in some samples, but never equal to zero.

with blood. Further research in the high-yielding dairy cow during the period of insemination is needed to investigate the origin of the distinct FFA composition in the follicular fluid compared with blood.

The steroid composition of dominant follicle fluid from the normal and delayed first ovulating postpartum cow groups was not different. However, the amounts of E_2 and P_4 varied considerably between follicles. Before induction of the LH peak, the E_2 level in follicular fluid is a measure of follicle activity, in which a high level of E_2 indicates a healthy follicle (Ginther et al., 1997; Austin et al., 2001). Upon LH activation, the level of P_4 in the periovulatory follicle increases at the expense of E_2, resulting in a low E_2:P_4 ratio (Dieleman et al., 1983). Competent oocytes, which are able to develop into blastocysts after fertilization, originate from periovulatory follicles with levels of E_2 $\leq$0.25 μM and P_4 $\geq$0.26 μM (Aardema et al., 2013b). In the non-periovulatory stage, a low E_2:P_4 ratio indicates an aberrant inactive dominant follicle (Ireland and Roche, 1982). A lower E_2:P_4 ratio in follicular fluid correlated with higher FFA levels. The FFA level was approximately 2.5 times higher when the E_2:P_4 ratio in follicular fluid was $<$1 compared with an E_2:P_4 ratio $>$2. The higher FFA level in follicular fluid with low E_2:P_4 can be explained by the more pronounced accumulation of all FFA fractions and, in particular, the unsaturated FFA. The differential degree of FFA accumulation in the dominant follicle with low E_2:P_4 ratio is not explained by the degree of NEB or by the timing of the first postpartum ovulation. Furthermore, the low E_2:P_4 ratio was not related to follicular wall deterioration because the levels were 2 to 4 times higher than those observed in blood, indicating that the blood–follicle barrier was intact and that the relatively high FFA levels in follicles with a low E_2:P_4 ratio, compared with follicles with a higher E_2:P_4 ratio, were not due to damage of the blood–follicle barrier. Previously, elevated levels in follicular fluid during an experimentally induced NEB were associated with a reduced concentration of E_2 in dominant follicles and elevated FFA levels (Jorritsma et al., 2003). Another study demonstrated reduced cell proliferation and increased apoptosis in granulosa cells after in vitro exposure to elevated FFA levels, together with increased E_2 concentrations (Vanholder et al., 2005). The findings of Vanholder et al. (2005) indicate a negative effect of elevated FFA levels on the survival of granulosa cells, but the effect on the E_2 production is more complex. Apart from elevated FFA levels, other factors may affect the survival and E_2 production of granulosa cells. For example, the commonly reduced levels of IGF-1 during NEB appear to be negatively associated with follicular growth and E_2 concentration (Beam and Butler, 1998; Wathes et al., 2007). Elevated FFA levels in combination with a low E_2:P_4 ratio in the dominant follicles may be due to a distinct FFA metabolism in this type of follicle. Future research should establish whether elevated FFA levels could be used as a predictive marker for the quality of the dominant follicle.

CONCLUSIONS

An overall elevation in circulating FFA concentrations during the early postpartum period is associated with delayed ovulation. However, the FFA composition of the follicular fluid of dominant follicles and in the blood at d 55, 80, and 105 postpartum appears to be similar in cows with delayed ovulation and in those that ovulate before d 35. The marked differences in the FFA composition of follicular fluid and blood indicate that these follicles had functional blood–follicle barriers and suggest the potential of follicles to selectively take up, accumulate, or metabolize desired fatty acids. We found no indications for a reflection of the elevated FFA levels early postpartum in the follicular fluid of dominant follicles during the preferred period of insemination that could explain the apparent link between NEB and reduced fertility due to potential harm by FFA to the oocyte. Interestingly, regardless of normal and delayed first ovulation and time postpartum, the E_2:P_4 ratio in follicular fluid correlated with FFA levels, with a low E_2:P_4 ratio being associated with a high FFA level. The relevance of this observation needs to be further investigated because it is a potential detection method for aberrant follicles.

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